

## FINAL REPORT

**Test Facility Study No. 511880**

### **Evaluation of the Ability of MLA-3202 to Induce Chromosome Aberrations in Cultured Peripheral Human Lymphocytes**

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**15 September 2016**

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**1. STATEMENT OF GLP COMPLIANCE**

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands

All phases of this study performed by the test facility were conducted in compliance with the following GLP regulations:

- OECD Principles of Good Laboratory Practice concerning Mutual Acceptance of Data in the Assessment of Chemicals, 26 November 1997 (C(97) 186 Final);
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

Except for the following:

- The test item characterisation information supplied by the sponsor was produced under the sponsor's quality system.

Analysis of test item in vehicle for concentration, stability, homogeneity was not performed, however, to limit the impact, the test item preparation was performed with approved procedures and documented in detail. Preparations were visually inspected for homogeneity prior to use and all preparations were used within 2 hours after preparation of the formulation.

The data generated and reported are considered to be valid.

Charles River Den Bosch



Signature: .....

Name: I.A.J. Verbaan, PhD.

Title: Study Director

Date: ...15 September 2016...

**2. TEST FACILITY QUALITY ASSURANCE STATEMENT**

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands.

Study title: Evaluation of the ability of MLA-3202 to induce chromosome aberrations in cultured peripheral human lymphocytes

This report was inspected by the Charles River Den Bosch Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s).

The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

**Project** 511880

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
<b>Study</b>	Study Plan Report	25-Mar-2016 21-Jul-2016	25-Mar-2016 22-Jul-2016	25-Mar-2016 22-Jul-2016
<b>Process</b>	<b>Genetic and In Vitro Toxicology</b> Test Substance Handling Exposure Observations/Measurements Specimen Handling	22-Mar-2016	31-Mar-2016	04-Apr-2016
	<b>Test Substance Receipt</b> Test Substance Handling	09-May-2016	20-May-2016	24-May-2016

The facility inspection program is conducted in accordance with Standard Operating Procedure.

The review of the final report was completed on the date of signing this QA statement.

Charles River Den Bosch

Signature: ..... Bouhuizen .....

Name: Ali Bouhuizen M. Sc.  
Compliance Specialist III

Date: ..... 15-Sep-2016 .....

### 3. SUMMARY

Evaluation of the ability of MLA-3202 to induce chromosome aberrations in cultured peripheral human lymphocytes.

This report describes the effect of MLA-3202 on the number of chromosome aberrations in cultured peripheral human lymphocytes in the presence and absence of a metabolic activation system (phenobarbital and  $\beta$ -naphthoflavone induced rat liver S9-mix). The possible clastogenicity of MLA-3202 was tested in two independent experiments.

The study procedures described in this report are in compliance with the most recent OECD guideline.

Batch RC-1045 of MLA-3202 was a clear amber red liquid. The test item was dissolved in dimethyl sulfoxide.

In the first cytogenetic assay, MLA-3202 was tested up to 164  $\mu\text{g}/\text{ml}$  for a 3 h exposure time with a 24 h fixation time in the absence and presence of 1.8% (v/v) S9-fraction. MLA-3202 precipitated in the culture medium at this dose level.

In the second cytogenetic assay, MLA-3202 was tested up to 70  $\mu\text{g}/\text{ml}$  for a 24 h continuous exposure time with a 24 h fixation time and up to 50  $\mu\text{g}/\text{ml}$  for a 48 h continuous exposure time with a 48 h fixation time in the absence of S9-mix. Appropriate toxicity was reached at these dose levels.

The number of cells with chromosome aberrations found in the solvent control cultures was within the 95% control limits of the distribution of the historical negative control database. Positive control chemicals, mitomycin C and cyclophosphamide, both produced a statistically significant increase in the incidence of cells with chromosome aberrations. In addition, the number of cells with chromosome aberrations found in the positive control cultures was within the 95% control limits of the distribution of the historical positive control database. It was therefore concluded that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

MLA-3202 did not induce any statistically significant and/or biologically relevant increase in the number of cells with chromosome aberrations in the absence and presence of S9-mix, in either of the two independently performed experiments.

No effects of MLA-3202 on the number of polyploid cells and cells with endoreduplicated chromosomes were observed both in the absence and presence of S9-mix. Therefore it can be concluded that MLA-3202 does not disturb mitotic processes and cell cycle progression and does not induce numerical chromosome aberrations under the experimental conditions described in this report.

Finally, it is concluded that this test is valid and that MLA-3202 is not clastogenic in human lymphocytes under the experimental conditions described in this report.

#### 4. INTRODUCTION

Due to the acquisition of WIL Research by Charles River, the name of the WIL Research facility in Den Bosch, has been changed to Charles River Laboratories Den Bosch BV, Hambakenwetering 7, 5231 DD 's Hertogenbosch, The Netherlands. Study documents may contain both names and both names are considered equivalent and may be used as the name of WIL Research transitions to Charles River.

##### 4.1. Study schedule

Experimental starting date : 29 March 2016

Experimental completion date : 09 June 2016

##### 4.2. Purpose

The objective of this study was to evaluate MLA-3202 for its ability to induce structural chromosome aberrations in cultured human lymphocytes (1), either in the presence or absence of a metabolic activation system (S9-mix).

##### Background of the test system

Whole blood samples obtained from healthy subjects were treated with an anti-coagulant (heparin) and cultured in the presence of a mitogen (phytohaemagglutinin). These stimulated human lymphocytes were used because they are sensitive indicators of clastogenic activity of a broad range of chemicals (1-5).

The stimulated lymphocytes were exposed to MLA-3202 both in the absence and presence of a metabolic activation system (S9-mix). In combination with this metabolic activation system indirect chemical mutagens, i.e. those requiring metabolic transformation into reactive intermediates, can be tested for possible clastogenic effects *in vitro*.

At predetermined intervals after exposure of the stimulated human lymphocytes to MLA-3202, cell division was arrested in the metaphase stage of the cell cycle by addition of the metaphase-arresting chemical colchicine. Cells were harvested, stained and metaphase cells were analysed for the presence of structural chromosome aberrations such as breaks, gaps, minutes, dicentrics and exchange figures. Results from cultures treated with MLA-3202 were compared with control (vehicle) treated cultures.

Chromosome aberrations are generally evaluated in the first post-exposure mitosis (i.e. 24 hours after exposure). However, since the appearance of the first post-exposure mitosis could be considerably delayed due to toxic insult to the cells, cells were also harvested 48 hours after exposure to cover the interval in which maximum aberration frequency was expected.

A test item that induces a positive response in this assay is presumed to be a potential mammalian cell clastogenic agent.

##### 4.3. Guidelines

The study procedures described in this report are in compliance with the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Guideline no. 473: *In Vitro Mammalian Chromosome Aberration Test* (adopted September 26, 2014).

**4.4. Retention of records and materials**

Records and material pertaining to the study, which include study plan and amendments, raw data, specimens, except perishable specimens, and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

Perishable specimens (e.g. requiring refrigeration or freezing) will be discarded following evaluation in the study without further notice to the study sponsor.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed.

**4.5. Responsible personnel****4.5.1. Test facility**

Study Director I.A.J. Verbaan, PhD.

**4.5.2. Sponsor Representative**

Study Monitor Audrey Batoon, Ph.D.

**5. MATERIALS AND METHODS****5.1. Test item****5.1.1. Test item information**

Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

For Certificate of Analysis, see [APPENDIX 7](#).

**5.1.2. Study specific test item information**

Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Stability at higher temperatures	Stable
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3

**5.2. Vehicle information**

Solubility in vehicle	Dimethyl sulfoxide: Not indicated
Stability in vehicle	Dimethyl sulfoxide: Not indicated

**5.3. Reference item****5.3.1. Negative control**

The vehicle for the test item was dimethyl sulfoxide (DMSO; SeccoSolv, Merck, Darmstadt, Germany).

### 5.3.2. Positive controls

Without metabolic activation (-S9-mix):

Mitomycin C (MMC-C; CAS no. 50-07-7, Sigma, Zwijndrecht, The Netherlands) was used as a direct acting mutagen at a final concentration of 0.5 and 0.75 µg/ml for a 3 h exposure period, 0.2 and 0.3 µg/ml for a 24 h exposure period and 0.1 and 0.15 µg/ml for a 48 h exposure period.

With metabolic activation (+S9-mix):

Cyclophosphamide (CP; CAS no. 50-18-0. Baxter B.V., Utrecht, The Netherlands) was used as an indirect acting mutagen, requiring metabolic activation, at a final concentration of 10 µg/ml for a 3 h exposure period (24 h fixation time).

Solvent for positive controls:

Hanks' Balanced Salt Solution (HBSS) (Life technologies, Bleiswijk, The Netherlands), without calcium and magnesium.

All reference stock solutions were stored in aliquots at ≤-15°C in the dark. These solutions were thawed immediately before use.

### 5.4. Test item preparation

No correction was made for the purity/composition of the test compound.

A solubility test was performed. MLA-3202 was dissolved dimethyl sulfoxide of spectroscopic quality.

The final concentration of the solvent in the culture medium was 1.0% (v/v).

### 5.5. Test system

Cultured peripheral human lymphocytes were used as test system. Peripheral human lymphocytes are recommended in international guidelines (OECD).

Blood was collected from healthy adult, non-smoking volunteers (approximately 18 to 35 years of age). The Average Generation Time (AGT) of the cells and the age of the donor at the time the AGT was determined (December 2014) are presented below:

Dose range finding study/First cytogenetic assay:      age 27, AGT = 13.5 h  
First cytogenetic assay (24 hours incubation period):    age 24, AGT = 12.4 h  
Second cytogenetic assay (48 hours incubation period): age 28, AGT = 13.4 h

### 5.6. Cell culture

#### Blood samples

Blood samples were collected by venepuncture using the Venoject multiple sample blood collecting system with a suitable size sterile vessel containing sodium heparin (Vacutette, Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Immediately after blood collection lymphocyte cultures were started.

#### Culture medium

Culture medium consisted of RPMI 1640 medium (Life technologies), supplemented with 20% (v/v) heat-inactivated (56°C; 30 min) foetal calf serum (Life technologies), L-glutamine (2 mM) (Life technologies), penicillin/streptomycin (50 U/ml and 50 µg/ml respectively) (Life technologies) and 30 U/ml heparin (Sigma, Zwijndrecht, The Netherlands).

### Lymphocyte cultures

Whole blood (0.4 ml) treated with heparin was added to 5 ml or 4.8 ml culture medium (in the absence and presence of S9-mix, respectively). Per culture 0.1 ml (9 mg/ml) phytohaemagglutinin (Remel, Europe Ltd., Dartford, United Kingdom) was added.

### Environmental conditions

All incubations were carried out in a controlled environment, in which optimal conditions were a humid atmosphere of 80 - 100% (actual range 56 - 89%), containing  $5.0 \pm 0.5\%$  CO<sub>2</sub> in air in the dark at  $37.0 \pm 1.0^\circ\text{C}$  (actual range 34.9 - 37.1°C). Temperature and humidity were continuously monitored throughout the experiment. The CO<sub>2</sub> percentage was monitored once on each working day. Temporary deviations from the temperature, humidity and CO<sub>2</sub> percentage may occur due to opening and closing of the incubator door. Based on laboratory historical data these deviations are considered not to affect the study integrity.

### **5.7. Metabolic activation system**

Rat S9 homogenate was obtained from Trinova Biochem GmbH, Giessen, Germany and is prepared from male Sprague Dawley rats that have been dosed orally with a suspension of phenobarbital (80 mg/kg body weight) and β-naphthoflavone (100 mg/kg).

#### **5.7.1. Preparation of S9-mix**

S9-mix was prepared immediately before use and kept on ice. S9-mix components contained per ml physiological saline: 1.63 mg MgCl<sub>2</sub>.6H<sub>2</sub>O (Merck); 2.46 mg KCl (Merck); 1.7 mg glucose-6-phosphate (Roche, Mannheim, Germany); 3.4 mg NADP (Randox Laboratories Ltd., Crumlin, United Kingdom); 4 μmol HEPES (Life technologies).

The above solution was filter (0.22 μm)-sterilized. To 0.5 ml S9-mix components 0.5 ml S9-fraction was added (50% (v/v) S9-fraction) to complete the S9-mix.

Metabolic activation was achieved by adding 0.2 ml S9-mix to 5.3 ml of a lymphocyte culture (containing 4.8 ml culture medium, 0.4 ml blood and 0.1 ml (9 mg/ml) phytohaemagglutinin). The concentration of the S9-fraction in the exposure medium was 1.8% (v/v).

### **5.8. Study design**

#### **5.8.1. Dose range finding test / First cytogenetic assay**

In order to select the appropriate dose levels for the chromosome aberration test cytotoxicity data were obtained in a dose range finding test. MLA-3202 was tested in the absence and in the presence of 1.8% (v/v) S9-fraction.

Lymphocytes (0.4 ml blood of a healthy donor was added to 5 ml or 4.8 ml culture medium, without and with metabolic activation respectively and 0.1 ml (9 mg/ml) Phytohaemagglutinin) were cultured for 48 h and thereafter exposed to selected doses of MLA-3202 for 3 h, 24 h and 48 h in the absence of S9-mix or for 3 h in the presence of S9-mix. A negative control was included at each exposure time.

The highest tested concentration was determined by the solubility of MLA-3202 in the culture medium.

The test item precipitated at concentrations of 164 μg/ml and upwards. The lymphocytes were cultured in duplicate at the 3 h exposure time and appropriate positive controls were included. The cytogenetic assay was carried out as described by Evans, 1984 (2) with minor modifications. MLA-3202 was tested in the absence and presence of 1.8% (v/v) S9-fraction in duplicate.

After 3 h exposure to MLA-3202 in the absence or presence of S9-mix, the cells were separated from the exposure medium by centrifugation (5 min, 365 g). The supernatant was removed and cells were rinsed with 5 ml HBSS. After a second centrifugation step, HBSS was removed and cells were re-suspended in 5 ml culture medium and incubated for another 20 - 22 h (24 h fixation time). The cells that were exposed for 24 h and 48 h in the absence of S9-mix were not rinsed after exposure but were fixed immediately (24 h and 48 h fixation time).

Cytotoxicity of MLA-3202 in the lymphocyte cultures was determined using the mitotic index. No cytotoxicity was observed in the duplicate cultures of the 3 h exposure time and the slides were scored for chromosome aberrations. The pilot study (short term exposure period) was used as the first cytogenetic assay.

Based on the results of the dose range finding test an appropriate range of dose levels was chosen for the second cytogenetic assay considering the highest dose level was determined by the solubility. As clear negative results were obtained in the presence of metabolic activation, the repetition of the experiment was not considered necessary.

#### **5.8.2. Second cytogenetic assay**

To confirm the results of the first cytogenetic assay a second cytogenetic assay was performed with an extended exposure time of the cells in the absence of S9-mix.

Lymphocytes were cultured for  $48 \pm 2$  h and thereafter exposed in duplicate to selected doses of MLA-3202 for 24 h and 48 h in the absence of S9-mix.

The cells were not rinsed after exposure but were fixed immediately after 24 h and 48 h (24 h and 48 h fixation time). Appropriate negative and positive controls were included in the second cytogenetic assay. Initially the 48 h exposure was rejected due to technical reasons, this part of the study was repeated.

#### **5.8.3. Chromosome preparation**

During the last 2.5 - 3 h of the culture period, cell division was arrested by the addition of the spindle inhibitor colchicine (0.5 µg/ml medium) (Acros Organics, Geel, Belgium). Thereafter the cell cultures were centrifuged for 5 min at 365 g and the supernatant was removed. Cells in the remaining cell pellet were swollen by a 5 min treatment with hypotonic 0.56% (w/v) potassium chloride (Merck) solution at 37°C. After hypotonic treatment, cells were fixed with 3 changes of methanol (Merck): acetic acid (Merck) fixative (3:1 v/v).

#### **5.8.4. Preparation of slides**

Fixed cells were dropped onto cleaned slides, which were immersed in a 1:1 mixture of 96% (v/v) ethanol (Merck)/ether (Merck) and cleaned with a tissue. The slides were marked with the Charles River Den Bosch study identification number and group number. At least two slides were prepared per culture. Slides were allowed to dry and thereafter stained for 10 - 30 min with 5% (v/v) Giemsa (Merck) solution in Sörensen buffer pH 6.8. Thereafter slides were rinsed in water and allowed to dry.

The dry slides were automatically embedded in a 1:10 mixture of xylene (Klinipath, Duiven, The Netherlands)/pertex (Histolab, Gothenburg, Sweden) and mounted with a coverslip in an automated cover slipper (Leica Microsystems B.V., Rijswijk, The Netherlands).

#### **5.8.5. Mitotic index/dose selection for scoring of the cytogenetic assay**

The mitotic index of each culture was determined by counting the number of metaphases from at least 1000 cells (with a maximum deviation of 5%). At least three analysable concentrations were used for scoring of the cytogenetic assay. Chromosomes of metaphase

spreads were analysed from those cultures with an inhibition of the mitotic index of  $55 \pm 5\%$ , whereas the mitotic index of the lowest dose level was approximately the same as the mitotic index of the solvent control. Also cultures treated with an intermediate dose were examined for chromosome aberrations. For the short term incubation period, the highest concentration analysed was determined by the solubility in the culture medium.

### **5.8.6. Analysis of slides for chromosome aberrations**

To prevent bias, all slides were randomly coded before examination of chromosome aberrations and scored. An adhesive label with Charles River Den Bosch study identification number and code was placed over the marked slide. One hundred and fifty metaphase chromosome spreads per culture were examined by light microscopy for chromosome aberrations. In case the number of aberrant cells, gaps excluded, was  $\geq 38$  in 75 metaphases, no more metaphases were examined. Only metaphases containing  $46 \pm 2$  centromeres (chromosomes) were analysed. The number of cells with aberrations and the number of aberrations were calculated. Since the lowest concentration of MMC-C resulted in a positive response the highest concentration was not examined for chromosome aberrations.

## **5.9. Interpretation**

### **5.9.1. Acceptability of the assay**

A chromosome aberration test is considered acceptable if it meets the following criteria:

- a) The concurrent negative control data are considered acceptable when they are within the 95% control limits of the distribution of the historical negative control database.
- b) The concurrent positive controls should induce responses that are compatible with those generated in the historical positive control database.
- c) The positive control item induces a statistically significant increase in the number of cells with chromosome aberrations. The positive control data will be analysed by the Fisher's exact test (one-sided,  $p < 0.05$ ).

### **5.9.2. Data evaluation and statistical procedures**

Graphpad Prism version 4.03 (Graphpad Software, San Diego, USA) and ToxRat Professional (ToxRat Solutions<sup>®</sup> GmbH, Germany) were used for statistical analysis of the data.

A test item is considered positive (clastogenic) in the chromosome aberration test if:

- a) At least one of the test concentrations exhibits a statistically significant (Fisher's exact test, one-sided,  $p < 0.05$ ) increase compared with the concurrent negative control.
- b) Any of the results are outside the 95% control limits of the historical control data range.

A test item is considered negative (not clastogenic) in the chromosome aberration test if:

- a) None of the test concentrations exhibits a statistically significant (Fisher's exact test, one-sided,  $p < 0.05$ ) increase compared with the concurrent negative control.
- b) All results are inside the 95% control limits of the negative historical control data range.

In case the Fisher's exact test shows that there are statistically significant differences between one or more of the test item groups and the vehicle control group a Cochran Armitage trend test ( $p < 0.05$ ) will be performed to test whether there is a significant trend in the induction.

**5.10. List of deviations****5.10.1. List of study plan deviations**

There were no deviations from the study plan.

**5.10.2. List of standard operating procedures deviations**

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

**6. ELECTRONIC SYSTEMS FOR DATA ACQUISITION**

The following electronic systems were used for data acquisition:

REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): temperature and humidity.

**7. RESULTS****7.1. Dose range finding test / First cytogenetic assay**

At a concentration of 164 µg/ml MLA-3202 precipitated in the culture medium. At the 3 h exposure time, blood cultures were treated in duplicate with 17, 52 and 164 µg test item/ml culture medium with and without S9-mix (first cytogenetic assay).

At the 24 hour and 48 hour exposure time single blood cultures were treated with 5.4, 17, 52, 164 and 512 µg MLA-3202 /ml culture medium without S9-mix (dose range finding test).

[Table 1 \(APPENDIX 1\)](#) shows the mitotic index of cultures treated with various MLA-3202 concentrations or with the negative control item (dose range finding test).

[Table 2 \(APPENDIX 1\)](#) shows the mitotic index of cultures treated with various MLA-3202 concentrations or with the positive or negative control substances (first cytogenetic assay).

Both in the absence and presence of S9-mix, MLA-3202 did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations ([APPENDIX 1: Table 3, Table 4](#)).

Both in the absence and presence of S9-mix, MLA-3202 did not increase the number of polyploid cells and cells with endoreduplicated chromosomes.

**7.2. Second cytogenetic assay**

To obtain more information about the possible clastogenicity of MLA-3202, a second cytogenetic assay was performed in which human lymphocytes were continuously exposed to MLA-3202 in the absence of S9-mix for 24 or 48 hours. The following dose levels were selected for the second cytogenetic assay:

Without S9-mix : 10, 25, 40, 50, 60 and 70 µg/ml culture medium  
(24 and 48 h exposure time, 24 and 48 h fixation time).

[Table 5 \(APPENDIX 1\)](#) shows the mitotic index of cultures treated with various MLA-3202 concentrations or with the positive or negative control items.

Based on these observations the following doses were selected for scoring of chromosome aberrations:

Without S9-mix : 10, 50 and 70 µg/ml culture medium  
(24 h exposure time, 24 h fixation time).

10, 25 and 50 µg/ml culture medium  
(48 h exposure time, 48 h fixation time).

At the 24 h continuous exposure time MLA-3202 induced a statistically significant increase in the number of cells with chromosome aberrations at the highest tested concentration only ([APPENDIX 1; Table 6](#)). No significant trend was shown. In addition the increases were within the 95% control limits of the negative control historical data range. Therefore this increase is not considered biologically relevant.

At the 48 h continuous exposure time MLA-3202 did not induce a statistically significant increase in the number of cells with chromosome aberrations ([APPENDIX 1; Table 7](#)).

MLA-3202 did not induce a biologically relevant increase the number of polyploid cells and cells with endoreduplicated chromosomes. Although 1 endoreduplicated chromosome was observed at the highest concentration at the 48 hours exposure time, which is outside the 95% control limits of the distribution of the historical negative control database, this was considered an isolated event and therefore considered not biologically relevant.

### 7.3. Evaluation of the results

The ability of MLA-3202 to induce chromosome aberrations in human peripheral lymphocytes was investigated in two independent experiments. The highest concentration analysed was selected based on the solubility of the test item in the culture medium or on inhibition of the mitotic index of about 50% or greater

The mitotic indices of cultures treated with various MLA-3202 concentrations or with the negative control items are presented in [Table 1](#), [Table 2](#) and [Table 5 \(APPENDIX 1\)](#). The scores for the number of aberrant cells (gaps included and excluded) and the number of the various types of chromosome aberrations at the various concentrations of MLA-3202 are presented in [Table 3](#), [Table 4](#) and [Table 6 – 7 \(APPENDIX 1\)](#). Duplicate cultures are indicated by A and B. The criteria according to which the aberrations were classified are outlined in [APPENDIX 2](#). [APPENDIX 3](#) presents the statistical evaluations of the test results.

The number of cells with chromosome aberrations found in the solvent control cultures was within the 95% control limits of the distribution of the historical negative control database (see [APPENDIX 4](#)). The number of polyploid cells and cells with endoreduplicated chromosomes in the solvent control cultures was within the 95% control limits of the distribution of the historical negative control database (see [APPENDIX 6](#)). The positive control chemicals (MMC-C and CP) both produced statistically significant increases in the frequency of aberrant cells (see [APPENDIX 3](#)). In addition, the number of cells with chromosome aberrations found in the positive control cultures was within the 95% control limits of the distribution of the historical positive control database. It was therefore concluded that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

MLA-3202 did not induce any statistically significant and/or biologically relevant increase in the number of cells with chromosome aberrations in the absence and presence of S9-mix, in either of the two independently performed experiments.

No effects of MLA-3202 on the number of polyploid cells and cells with endoreduplicated chromosomes were observed both in the absence and presence of S9-mix. Therefore it can be concluded that MLA-3202 does not disturb mitotic processes and cell cycle progression and does not induce numerical chromosome aberrations under the experimental conditions described in this report.

## 8. CONCLUSION

Finally, it is concluded that this test is valid and that MLA-3202 is not clastogenic in human lymphocytes under the experimental conditions described in this report.

## 9. REFERENCES

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**APPENDIX 1****TABLES****Table 1****Mitotic index of human lymphocyte cultures treated with MLA-3202 in the dose range finding test**

MLA-3202 concentration ( $\mu\text{g/ml}$ )	<u>Number of metaphases</u>		
	Absolute	Number of cells scored	Percentage of control
<b>Without metabolic activation (-S9-mix)</b>			
24 h exposure time, 24 h fixation time			
Control <sup>a)</sup>	66	1020	100
5.4	64	1004	97
17	49	1015	74
52	25	1012	38
164 <sup>b)</sup>	0	1000	0
512 <sup>b)</sup>	c)	c)	c)
48 h exposure time, 48 h fixation time			
Control <sup>a)</sup>	31	1021	100
5.4	30	1010	97
17	27	1001	87
52	13	1000	42
164 <sup>b)</sup>	c)	c)	c)
512 <sup>b)</sup>	c)	c)	c)

a)Dimethyl sulfoxide

b)MLA-3202 precipitated in the culture medium.

c)Cell lysis

**Table 2**  
**Mitotic index of human lymphocyte cultures treated with MLA-3202 in the first**  
**cytogenetic assay**

MLA-3202 concentration ( $\mu\text{g/ml}$ )	<u>Number of metaphases<sup>a)</sup></u>					Percentage of control	
	Absolute		Number of cells scored				
<u>Without metabolic activation (-S9-mix)</u>							
3 h exposure time, 24 h fixation time							
Control <sup>b)</sup>	76	-	80	1000	-	1025	100
17	75	-	76	1025	-	1009	97
52	70	-	75	1010	-	1000	93
164 <sup>c)</sup>	41	-	37	1006	-	1007	50
MMC-C; 0.5 $\mu\text{g/ml}$	73	-	63	1006	-	1021	87
MMC-C; 0.75 $\mu\text{g/ml}$	62	-	57	1017	-	1017	76
<u>With metabolic activation (+S9-mix)</u>							
3 h exposure time, 24 h fixation time							
Control <sup>b)</sup>	75	-	79	1025	-	1029	100
17	71	-	69	1017	-	1021	91
52	70	-	70	1030	-	1030	91
164 <sup>c)</sup>	64	-	62	1000	-	1024	82
CP; 10 $\mu\text{g/ml}$	53	-	50	1008	-	1008	67

a) Duplicate cultures

b) Dimethyl sulfoxide

c) MLA-3202 precipitated in the culture medium.

**Table 3**

**Chromosome aberrations in human lymphocyte cultures treated with MLA-3202 in the absence of S9-mix in the first cytogenetic assay (3 h exposure time, 24 h fixation time)**

Conc	DMSO (1.0% v/v)			17 µg/ml			52 µg/ml			164 µg/ml			MMC-C 0.5 µg/ml		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index (%)	100			97			93			50			87		
No. of Cells scored	150	150	300	150	150	300	150	150	300	150	150	300	150	150	300
No. of Cells with aberrations (+ gaps) a)	0	0	0	0	0	0	0	0	0	0	0	0	28	25	53
No. of Cells with aberrations (- gaps)	0	0	0	0	0	0	0	0	0	0	0	0	27	24	51
g'													1	1	
g''															1
b'													14	12	
b''													5	6	
m'															
m''															
exch.													8	6	
dic															
d'															
misc.	poly									poly			p		
total aberr (+ gaps)	0	0		0	0		0	0		0	0		28	27	
total aberr (- gaps)	0	0		0	0		0	0		0	0		27	25	

a) Abbreviations used for various types of aberrations are listed in APPENDIX 2.

misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.

The numerical variation polypliody (poly) was not counted as an aberration.

\*) Significantly different from control group (Fisher's exact test), \* P < 0.05, \*\* P < 0.01 or \*\*\* P < 0.001.

**Table 4**  
**Chromosome aberrations in human lymphocyte cultures treated with MLA-3202 in the presence of S9-mix in the first cytogenetic assay (3 h exposure time, 24 h fixation time)**

Conc	DMSO (1.0% v/v)			17 µg/ml			52µg/ml			164 µg/ml			CP 10 µg/ml		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index (%)	100			91			91			82			67		
No. of Cells scored	150	150	300	150	150	300	150	150	300	150	150	300	150	150	300
No. of Cells with aberrations (+ gaps) a)	0	0	0	0	0	0	1	1	2	0	0	0	36	32	68
No. of Cells with aberrations (- gaps)	0	0	0	0	0	0	1	1	2	0	0	0	35	32	67
g'															1
g''															1
b'													23	21	
b''							1	1					9	5	
m'															
m''															
exch.													6	9	
dic															
d'															
misc.				2poly											
total aberr (+ gaps)	0	0		0	0		1	1		0	0		40	35	
total aberr (- gaps)	0	0		0	0		1	1		0	0		38	35	

a) Abbreviations used for various types of aberrations are listed in APPENDIX 2.35

misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.

The numerical variation polypliody (poly) was not counted as an aberration.

\*) Significantly different from control group (Fisher's exact test), \* P < 0.05, \*\* P < 0.01 or \*\*\* P < 0.001.

**Table 5**  
**Mitotic index of human lymphocyte cultures treated with MLA-3202 in the second cytogenetic assay**

MLA-3202 concentration ( $\mu\text{g/ml}$ )	<u>Number of metaphases<sup>a)</sup></u>			
	Absolute	Number of cells scored	Percentage of control	
<u>Without metabolic activation (-S9-mix)</u>				
24 h exposure time, 24 h fixation time				
Control <sup>b)</sup>	57 - 58	1010 - 1004	1004	100
10	55 - 51	1005 - 1031	1031	92
25	40 - 47	1016 - 1007	1007	76
40	40 - 41	1002 - 1004	1004	70
50	33 - 34	1022 - 1003	1003	58
60	32 - 33	1022 - 1009	1009	57
70 <sup>c)</sup>	26 - 26	1018 - 1019	1019	45
MMC-C; 0.2 $\mu\text{g/ml}$	40 - 45	1013 - 1032	1032	74
MMC-C; 0.3 $\mu\text{g/ml}$	33 - 39	1016 - 1015	1015	63
48 h exposure time, 48 h fixation time				
Control <sup>b)</sup>	45 - 38	1014 - 1000	1000	100
10	39 - 34	1000 - 1000	1000	88
25	29 - 26	1000 - 1000	1000	66
40	24 - 22	1000 - 1000	1000	55
50	19 - 19	1000 - 1000	1000	46
60 <sup>c)</sup>	13 - 15	1000 - 1000	1000	34
70 <sup>c)</sup>	18 - 15	1000 - 1000	1000	40
MMC-C; 0.1 $\mu\text{g/ml}$	40 - 34	1000 - 1000	1000	89
MMC-C; 0.15 $\mu\text{g/ml}$	33 - 31	1000 - 1000	1000	77

a) Duplicate cultures

b) Dimethyl sulfoxide

c) MLA-3202 precipitated in the culture medium

**Table 6**  
**Chromosome aberrations in human lymphocyte cultures treated with MLA-3202 in the absence of S9-mix in the second cytogenetic assay (24 h exposure time, 24 h fixation time)**

Conc	DMSO (1.0% v/v)			10 µg/ml			50 µg/ml			70 µg/ml			MMC-C 0.2 µg/ml		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index (%)	100			92			58			45			74		
No. of Cells scored	150	150	300	150	150	300	150	150	300	150	150	300	150	150	300
No. of Cells with aberrations (+ gaps) a)	0	0	0	0	5	5	2	3	5	4	2	6 <sup>*)</sup>	49	49	98 <sup>***)</sup>
No. of Cells with aberrations (- gaps)	0	0	0	0	5	5	2	3	5	3	2	5	49	48	97 <sup>***)</sup>
g'															1
g''															1
b'				5			2	1		3	2		33	41	
b''							2						15	12	
m'															
m''															
exch.													11	8	
dic															
d'															
misc.													3poly	poly	
total aberr (+ gaps)	0	0		0	5		2	3		4	2		59	62	
total aberr (- gaps)	0	0		0	5		2	3		3	2		59	61	

a) Abbreviations used for various types of aberrations are listed in APPENDIX 2. misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.

The numerical variation polyploidy (poly) was not counted as an aberration.

\*) Significantly different from control group (Fisher's exact test), \* P < 0.05, \*\* P < 0.01 or \*\*\* P < 0.001.

**Table 7**

**Chromosome aberrations in human lymphocyte cultures treated with MLA-3202 in the absence of S9-mix in the second cytogenetic assay (48 h exposure time, 48 h fixation time)**

Conc	DMSO (1.0% v/v)			10 µg/ml			25 µg/ml			50 µg/ml			MMC-C 0.1 µg/ml		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index (%)	100			88			66			46			89		
No. of Cells scored	150	150	300	150	150	300	150	150	300	150	150	300	100	100	200
No. of Cells with aberrations (+ gaps) a)	2	2	4	2	1	3	8	0	8	1	6	7	38	40	78
No. of Cells with aberrations (- gaps)	2	2	4	2	1	3	8	0	8	1	6	7	38	40	78
g'															
g''															
b'	2	1		1			7			1	4		26	26	
b''		1		1	1		1				1		17	14	
m'													2		
m''											2				
exch.						1							5	9	
dic															
d'															
misc.				poly						endo					
total aberr (+ gaps)	2	2		2	1		9	0		1	7		50	49	
total aberr (- gaps)	2	2		2	1		9	0		1	7		50	49	

a) Abbreviations used for various types of aberrations are listed in APPENDIX 2.

misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.

The numerical variations endoreduplication (endo) and polyploidy (poly) were not counted as an aberration.

\*) Significantly different from control group (Fisher's exact test), \* P < 0.05, \*\* P < 0.01 or \*\*\* P < 0.001.

**APPENDIX 2**  
**DEFINITIONS OF CHROMOSOME ABERRATIONS SCORED IN METAPHASE**  
**PORTRAITS**

Aberration	Abbreviation	Description
Chromatid gap	g'	An achromatic lesion which appears as an unstained region in the chromatid arm, the size of which is equal to or smaller than the width of the chromatid and the apparently "broken" segments of the chromatid arm are in alignment.
Chromosome gap	g"	An achromatic lesion which appears as an unstained region in both chromatids at the same position, the size of which is equal to or smaller than the width of the chromatid and the apparently "broken" segments of the chromatids are in alignment.
Chromatid break	b'	An achromatic lesion in a chromatid arm, the size of which is larger than the width of the chromatid. The broken segments of the chromatid arm are aligned or unaligned.
Chromosome break	b"	An achromatic lesion in both chromatids at the same position, the size of which is larger than the width of the chromatid. The broken segments of the chromatids are aligned or unaligned.
Chromatid deletion	d'	Deleted material at the end of a chromatid arm.
Minute	m'	A single, usually circular, part of a chromatid lacking a centromere.
Double minutes	m"	Two, usually circular, parts of a chromatid lacking a centromere.
Dicentric chromosome	dic	A chromosome containing two centromeres.
Tricentric chromosome	tric	A chromosome containing three centromeres.

**APPENDIX 2**  
**- continued -**

Aberration	Abbreviation	Description
Ring chromosome	r	A ring structure with a distinct lumen.
Exchange figure	exch.	An exchange(s) between two or more chromosomes resulting in the formation of a tri- or more-armed configuration.
Chromosome intrachange	intra	A chromosome intrachange is scored after rejoining of a lesion within one chromosome.
Pulverized chromosomes	p	A fragmented or pulverized chromosome
Multiple aberrations	ma	A metaphase spread containing ten or more of the above mentioned aberrations (chromatid and chromosome gaps not included). ma is counted as 10 aberrations.
Polyploidy	poly	A chromosome number that is a multiple of the normal diploid number.
Endoreduplication	endo	A form of polyploidy in which each centromere connects two or four pairs of chromatids instead of the normal one pair.

**APPENDIX 3**  
**STATISTICAL EVALUATION OF THE TEST RESULTS**

TOTAL NUMBER OF CELLS WITH ABERRATIONS; TREATMENT/CONTROL COMPARISON,

(INCLUSIVE/EXCLUSIVE GAPS).

EXPOSURE DOSE ( $\mu\text{g/ml}$ )	S9-MIX	GAPS	P-VALUE one-sided	DECISION AT 95% CONFIDENCE LEVEL
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**First cytogenetic assay (Fisher's exact test<sup>1)</sup>**

3 hours exposure time

*Positive controls*

MMC-C (0.5)	–	+	<0.0001	significant
	–	–	<0.0001	significant
CP (10)	+	+	<0.0001	significant
	+	–	<0.0001	significant

**Second cytogenetic assay (Fisher's exact test<sup>1)</sup>**

24 hours exposure time

*Positive control*

MMC-C (0.2)	–	+	<0.0001	significant
	–	–	<0.0001	significant

Test item

MLA-3202 (70)	–	+	=0.0305	significant
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48 hours exposure time

*Positive control*

MMC-C (0.1)	–	+	<0.0001	significant
	–	–	<0.0001	significant

**Second cytogenetic assay (24 hours exposure time; Cochran Armitage trend test)**

MLA-3202	–	+	=0.108	not significant
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<sup>1)</sup> Only statistically significant results are presented.

**APPENDIX 4**  
**HISTORICAL NEGATIVE CONTROL DATA FOR LYMPHOCYTE**  
**CHROMOSOME ABERRATION STUDIES**

	3 hours exposure time				24 hours exposure time		48 hours exposure time	
	Gaps included		Gaps excluded		Gaps included	Gaps excluded	Gaps included	Gaps excluded
	+ S9-mix	- S9-mix	+ S9-mix	- S9-mix	- S9-mix	- S9-mix	- S9-mix	- S9-mix
Mean number of aberrant cells per 100 cells	0.79	0.80	0.73	0.77	0.78	0.63	0.99	0.74
SD	1.03	1.11	1.03	1.12	1.08	1.02	1.24	1.13
n	256	254	256	254	250	250	248	248
Upper control limit (95% control limits)	3.32	3.21	3.21	3.10	3.20	2.75	4.06	3.12
Lower control limit (95% control limits)	-1.73	-1.62	-1.75	-1.57	-1.64	-1.48	-2.08	-1.64

SD = Standard deviation

n = Number of observations

Distribution historical negative control data from experiments performed between January 2012 and June 2016.

**APPENDIX 5**  
**HISTORICAL POSITIVE CONTROL DATA FOR LYMPHOCYTE CHROMOSOME**  
**ABERRATION STUDIES**

	3 hours exposure time				24 hours exposure time		48 hours exposure time	
	Gaps included		Gaps excluded		Gaps included	Gaps excluded	Gaps included	Gaps excluded
	+ S9-mix	- S9-mix	+ S9-mix	- S9-mix	- S9-mix	- S9-mix	- S9-mix	- S9-mix
Mean number of aberrant cells per 100 cells	33.08	29.34	32.44	28.90	30.61	29.71	35.57	34.54
SD	12.90	13.35	12.92	13.42	13.45	13.77	15.00	15.11
n	254	254	254	254	250	250	248	248
Upper control limit (95% control limits)	58.67	53.21	57.86	52.60	57.38	57.09	64.87	63.82
Lower control limit (95% control limits)	7.48	5.47	7.01	5.19	3.84	2.34	6.26	5.26

SD = Standard deviation

n = Number of observations

Distribution historical positive control data from experiments performed between January 2012 and June 2016.

**APPENDIX 6**  
**HISTORICAL NEGATIVE CONTROL DATA FOR NUMERICAL ABERRATIONS**  
**FOR LYMPHOCYTE CHROMOSOME ABERRATION STUDIES**

	3 hours exposure time				24 hours exposure time		48 hours exposure time	
	Poly		Endo		Poly	Endo	Poly	Endo
	+ S9-mix	- S9-mix	+ S9-mix	- S9-mix	- S9-mix	- S9-mix	- S9-mix	- S9-mix
Mean number numerical aberrations per 100 cells	0.07	0.08	0.01	0.02	0.08	0.01	0.09	0.01
SD	0.31	0.29	0.10	0.15	0.33	0.11	0.33	0.10
n	256	254	256	254	250	250	248	248
Upper control limit (95% control limits)	0.45	0.51	0.07	0.16	0.44	0.08	0.55	0.07
Lower control limit (95% control limits)	-0.31	-0.35	-0.05	-0.11	-0.28	-0.05	-0.37	-0.05

SD = Standard deviation

n = Number of observations

Poly = polyploidy

Endo = endoreduplication

Distribution historical negative control data from experiments performed between January 2012 and June 2016.

**APPENDIX 7**  
**CERTIFICATE OF ANALYSIS**



Chemtura Corporation  
12 Spencer St  
Naugatuck, CT 06770

Analytical Services  
[www.chemtura.com](http://www.chemtura.com)

**Certificate of Purity**

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C <sub>24</sub> H <sub>47</sub> NO <sub>3</sub>	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C <sub>22</sub> H <sub>45</sub> NO <sub>3</sub>	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C <sub>24</sub> H <sub>45</sub> NO <sub>3</sub>	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C <sub>24</sub> H <sub>49</sub> NO <sub>3</sub>	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C <sub>22</sub> H <sub>43</sub> NO <sub>3</sub>	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C <sub>26</sub> H <sub>45</sub> NO <sub>3</sub>	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C <sub>24</sub> H <sub>43</sub> NO <sub>3</sub>	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	C18:1 (oleic) acid
1.1	421.4	C <sub>26</sub> H <sub>47</sub> NO <sub>3</sub>	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

Blake Lewis                    3/7/16  
 Blake Lewis  
 Analytical REACH Scientist, Analytical Services  
 Date

Colin Moore                    3/7/16  
 Albert J. Nitowski  
 Sr. Technology Manager  
 Analytical and Lab Support Services  
 Date